# Cry9C Food Allergenicity Assessment Background Document

## I. The Agency's Rationale in Asking for Input

To complete its decision regarding the proposed expansion of the Cry9C plant-pesticide tolerance exemption, the Agency is seeking input regarding the safety of the Cry9C protein and similar stable proteins. On April 7, 1999, EPA announced the receipt of a pesticide petition from AgrEvo USA Company. The petition, 9F5050, proposed an amendment to 40 CFR 180.1192 to expand the current exemption from the requirement of a tolerance for *Bacillus thuringiensis* subspecies *tolworthi* Cry9C protein and the genetic material necessary for its production in corn. The proposed amendment would expand the tolerance exemption from corn used for feed only (and associated residues in meat, poultry, milk, or eggs resulting from animals fed such feed), to all food commodities. The EPA has reviewed all of the data submitted by AgrEvo USA to support its petition, as well as all other available scientific literature and has determined that several questions must be answered before the Agency can complete a risk assessment for all food commodities. Although none of the data supplied by AgrEvo suggests that the Cry9C protein is a food allergen, the Agency is seeking advise to determine the risk that exists from exposure to this protein based upon two of its biochemical characteristics (stability to heat and gastric digestion). These two biochemical characteristics are uncommon and separate Cry9C from the other Cry proteins seen to date as plant-pesticides.

The Agency is soliciting advice from experts on human food allergies, as well as the general public on: 1) EPA review of the data; and 2) the attached questions regarding assessment of allergenic potential of the protein for humans.

The Cry9C protein does not originate from an allergenic source and has no amino acid homology with known toxins or allergens in available protein databases. However, the Cry9C protein does not yet have a history of dietary exposure, is not readily digestible and has been shown to be stable at 90°C. To determine that a reasonable certainty of no harm will result from exposure to this protein and other introduced proteins with similar characteristics, EPA must determine if proteins with these biochemical characteristics are likely to affect the safety of a given food. One of the many parameters evaluated to ensure this safety finding is the potential allergenicity of the plant-pesticide.

Food allergies, like other common allergies, affect a small percentage of the population who may be sensitive to one or more specific proteins. Consumption of food containing these proteins can harm those who are sensitive with reactions ranging from gastrointestinal upset to, rarely, anaphylactic shock. For these sensitive persons, being able to identify and avoid the offending food is critical, including being able to identify the ingredients in processed food that may contain such ingredients. With the advent of genetic engineering of food crops, the introduction of novel proteins into the diet has become a reality. Currently, many of these novel proteins are introduced to control pests of food crops and are termed plant-pesticides.

## II. How the EPA Determined Allergenicity Risk to Proteins Originating From a

**Non-Allergen Source**

April 1994 Interagency Conference on Potential Allergenicity in Transgenic Food Crops

In April of 1994, EPA, the Food and Drug Administration, and the Department of Agriculture hosted a "Conference on Scientific Issues Related to Potential Allergenicity in Transgenic Food Crops." The goal of the Conference was to foster a dialogue among scientists on food allergies and those familiar with new varieties of food crops developed by gene transfer, to assess current information regarding what makes a substance, such as a protein, a food allergen and what means are available to assess the allergenic potential of a new protein.

The group of thirteen invited scientists included Dr. Robert Aalberse, Univ. of Amsterdam, The Netherlands; Dr. Martin Chapman, Univ. of Virginia; Dr. Peter Day, Rutgers; Dr. Kenneth Frey, Iowa State Univ.; Dr. Michael Kemeny, Guy's Hospital, London; Dr. T.P. King, Rockefeller Univ.; Dr. Yoichi Kohno, Chiba Univ., Japan; Dr. Samuel Lehrer, Tulane Univ.; Dr. Dean Metcalf, NIAID/NIH; Dr. Marshall Plaut, NIH; Dr. Richard Raybourne, CFSAN/FDA; Dr. Steven Taylor, Univ. of Nebraska; and Dr. John Yunginger, Mayo Clinic.

The invited scientists presented and discussed papers on plant breeding and biotechnology, allergenic foods, exposure and allergenic response, T cell and B cell antigenic determinants, *in vitro* and *in vivo* diagnostics, and animal models. The scientists noted: (1) that allergenic reactions to foods occur in a small percentage of the population, but nevertheless, affect a significant number of individuals; (2) life threatening reactions are a rare occurrence, and most allergic reactions to foods can be attributed to less than a dozen foods, e.g., milk, eggs, fish, crustaceans, mollusks, tree nuts, wheat and legumes; (3) methods are available to assess allergenic potential for proteins that are derived from sources to which consumers have reacted and for which serum is available, but it may be useful to establish a serum bank; (4) there are no direct methods to assess potential allergenicity of proteins from sources that are not known to produce food allergy; (5) although, some assurance can be provided to minimize the likelihood that a new protein will cause an allergic reaction by evaluating its similarity with characteristics of known food allergens (i.e. whether the new protein has a similar protein sequence, is prevalent in food, is resistant to enzymatic and acid degradation, is heat stable, and is of the appropriate molecular size), no single factor is predictive; and (6) glycosylation of the protein is not considered a useful parameter for predicting allergenic potential.

History of Plant-Pesticides Evaluated for Use in Human Food and/or Animal Feed

All plant-pesticides that have been approved for use in food and feed to date have originated from sources not known to be food allergens and thus were not expected to be food allergens. The following chart presents a list of the proteins in plant-pesticides that have been approved for direct human consumption in food as of September 1999.

| **Plant-Pesticide Protein** | **Approved Dietary Use** | **40 CFR Citation** |
|---|---|---|
| Watermelon Mosaic Virus-2 Coat Protein | All Food Commodities | 180.1184 |

| | | |
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| Zucchini Yellow Mosaic Virus Coat Protein | All Food Commodities | 180.1184 |
| Potato Virus Y Coat Protein | All Food Commodities | 180.1182 |
| Papaya Ringspot Virus Coat Protein | All Food Commodities | 180.1185 |
| Cucumber Mosaic Virus Coat Protein | All Food Commodities | 180.1186 |
| Potato Leaf Roll Virus Replicase Gene | All Food Commodities | 180.1183 |
| *Bacillus thuringiensis Cry3A* Protein | Potatoes | 180.1147 |
| *Bacillus thuringiensis Cry1Ac Protein* | All Plant Raw Agricultural Commodities | 180.1155 |
| *Bacillus thuringiensis Cry1Ab Protein* | All Plant Raw Agricultural Commodities | 180.1173 |
| *Bacillus thuringiensis Cry9C Protein* | Corn Used for Feed; As Well As Meat, Poultry, Milk, or Eggs Resulting From Animals Fed Such Feed | 180.1192 |

Viral Proteins

For the viral coat proteins and the potato leaf roll virus replicase gene, EPA's allergenicity assessments were based on two main factors. These nucleic acids and proteins did not come from known allergenic sources and dietary exposure to these viral nucleic acids and proteins would not significantly change with the use of the plant-pesticides. The viruses that produce these nucleic acids and proteins are ubiquitous in the agricultural environment, and in virus-infected plants, express the nucleic acids and proteins at levels higher than in transgenic plants. Food derived from virus-infected plants have historically been a part of the human and domestic animal diet, with no observed adverse effects to human health, including that of infants and children, upon consumption.

*Bacillus thuringiensis* Cry Proteins

For review of the Cry1Ab, Cry1Ac, Cry3A, and Cry9C *Bacillus thuringiensis* proteins, the EPA used the approach outlined in the April 1994 Interagency Conference and described in the paper, Assessment of the Allergenic Potential of Foods Derived from Genetically Engineered Crop Plants *Critical Reviews in Food Science and Nutrition*, 36(s):S165-S1866 (1996, CRC Press, Inc.). Additional factors considered for the Cry1Ab, Cry1Ac, and Cry3A included: (1) lack of known allergic reactions from exposure to these proteins which were already present in the human diet from Bt sprayable formulations; (2) a lack of amino acid similarity to known protein allergens; and (3) relatively rapid break down of the proteins upon exposure to gastric digestion .

The amino acid homology analyses were useful in supporting the assumption that the plant-pesticides were not structurally similar to proteins known to be allergens or mammalian toxins. None of the *Bacillus thuringiensis* proteins found in the plant-pesticides listed above were shown to be similar to proteins known to be allergens or mammalian toxins.

The *in vitro* digestibility studies predicted the fate of the proteins in the human digestive system (stomach and intestine). This is helpful in searching for potential food allergens because many food allergens are stable to the proteolytic and acidic conditions of human digestive systems and such stable proteins have an increased chance of reaching the intestines, where many food allergens elicit their response. The Cry1Ab, Cry1Ac, and Cry3A proteins were all quickly degraded by low pH and gastric enzymes under test conditions. Some Cry proteins expressed in plants have been shown to be present in the raw agricultural commodity but are broken down by processing into feed pellets. Many food allergens, such as those in soybeans and peanuts, are resistant to the heat of food processing and are found in processed foods.

EPA determined that the Cry1Ab, Cry1Ac, and Cry3A proteins had a reasonable certainty of not causing harm as food allergens since they triggered none of the criteria mentioned above. In addition, the proteins produced in the plants were non-glycosylated. The Agency recognizes glycosylation is not necessarily a useful parameter for predicting allergenic potential of a new protein. However, glycosylation and other post-translational modifications can be used to discern potential alterations due to plant expression of the introduced protein. Glycosylation of the plant expressed protein is also critical in establishing test substance equivalency when sources other than plant protein are used as the test substance for toxicity testing. Review of the Cry9C protein included the same parameters listed for the other Cry proteins described above. Like those proteins, Cry9C does not share amino acid similarity or protein structural similarity to proteins known to be allergens or mammalian toxins. However, unlike the other Cry proteins reviewed, Cry9C is stable when exposed to simulated gastric digestion and to temperatures at 90°C. Two of the Cry9C protein's biochemical features (stability to simulated gastric digestion and to temperatures at 90°C) alter the weight of evidence argument typically used by EPA for assessing the food allergenicity of an introduced protein. The Agency is therefore taking this opportunity to revisit the food allergenicity issue with experts in the field and determine if any new information has come forth since the 1994 Conference in Annapolis, MD.

The EPA has determined that the Cry9C protein would not likely cause an allergic reaction to man when used in feed corn because: 1) it was not from allergenic sources and 2) the best available information indicates that edible products derived from animals such as meat, milk and eggs, intended for human consumption, have not been shown to be altered in their allergenicity due to changes in the feed stock utilized. For example, despite the fact that soybeans contain important clinical allergenic proteins that are stable to gastric fluids and high temperatures, the allergenic properties are not transferred into the meat, milk or eggs of animals fed soybean products and thus, show no adverse effects when eaten by soybean-sensitive individuals.

## III. Summary of EPA's Data Evaluation Records (of AgrEvo Submitted Data) and Preliminary Risk Assessment Regarding Human Consumption of Cry9C Corn

The classifications that are found for each data submission are assigned by the EPA science reviewer and are an indication of the usefulness of the information contained in the documents and meets the intent of the test guidelines. A rating of "ACCEPTABLE" indicates the data is useful for risk assessment, is scientifically valid and has been satisfactorily performed according to

accepted EPA guidelines or other justified criteria. A "SUPPLEMENTAL" rating indicates the data provide some information that can be useful for risk assessment. However, " SUPPLEMENTAL" studies may either have certain aspects not adequately described to be scientifically acceptable (SUPPLEMENTAL. UPGRADEABLE) or have not been done to fulfill a specific EPA guideline requirement. If a study is rated as "SUPPLEMENTAL. UPGRADEABLE," there is always an indication of what is lacking or what can be provided to change the rating to "ACCEPTABLE." If there is simply a "SUPPLEMENTAL" rating, the reviewer will often state that the study is not required by current EPA guidelines or does not need to be reclassified as "ACCEPTABLE."

**Safety Assessment of StarLink Genetically Modified Corn, Containing the Truncated *Bt* Insecticidal Protein Cry9C for Human Food Use - MRID No. 447140-01**

The summary information contained in this submission is intended to support the belief by AgrEvo that the Cry9C protein, and the StarLink corn plants expressing this protein, do not pose a significant risk to human health. Some of the data and information provided by AgrEvo is compelling and supportive of the belief of no significant risk. However, EPA believes that at least an equal amount of data and information is either inconclusive, or indicates that Cry9C exhibits some characteristics of known allergens. Except for the reviews by BIBRA International, Dr. Andrew Cockburn and Dr. Samuel B. Lehrer, all of the other information in MRID No. 447140-01 has been submitted previously, or under different MRID numbers. Several of the studies enclosed in this submission also require supplementary information or explanation.

As with much of the data and information submitted previously and with this package, it is not possible to reach a definite conclusion regarding the allergenic potential of the Cry9C protein based upon the supplementary information. As can be seen from the enclosed letter and reviews, even expert analysis of potential allergenicity of this protein differs. Based upon these factors, it is not possible for the Agency to determine that there is a lack of allergenic potential from Cry9C based upon the available information. Further data and clarification must be provided to aid in such a determination.
CLASSIFICATION: SUPPLEMENTAL. This report is a summary of data and other information submitted by AgrEvo and does not provide additional information not found in the data packages submitted in support of this application.

**Bt Cry9C Protein: Investigative Study of the Potential for Binding to Mouse Intestinal Brush Border Membrane Vesicles - MRID No. 447343-01**

The addition of unlabeled Cry9C displaced labeled (biotinylated) Cry9C on intestinal brush border membrane vesicles (BBMV) of European corn borer, *Ostrinia nubilalis*. AgrEvo claims this study demonstrates that the Cry9C binds specifically and saturably to *Ostrinia nubilalus* BBMVs. The addition of unlabeled Cry9C did not result in the displacement of the labeled Cry9C in preparations of mouse intestinal BBMVs. AgrEvo claims this demonstrates that Cry9C shows only a small amount of non-specific binding, and does not bind specifically and saturably to mouse intestinal BBMVs.

However, the copy of the electrophoretogram provided is not of very good quality, and therefore, it is impossible for the Agency to reach these same conclusions. Assuming that clear figures are available, it is also unclear to the Agency what AgrEvo means by "displacement" by the unlabeled Cry9C in the insect BBMV samples. The term "displacement" indicates an active process where there is a mechanism responsible for dislodging labeled proteins and allowing these displaced proteins to be replaced by the unlabeled Cry9C proteins, or some similar process. There is no discussion in this submission which addresses the displacement issue. Further explanation/discussion should be provided.

CLASSIFICATION: SUPPLEMENTAL. This submission can be upgraded to ACCEPTABLE with submission of adequate figures and additional information to support the conclusion reached regarding the binding potential of Cry9C to the tissues tested. Supplemental data/information should include:

1. Adequate figures (originals or high quality reproductions) of the chemiluminescent results that will allow for clear differentiation between protein signals and background "noise".
2. Molecular weight markers to clearly identify the molecular weights of the identified protein bands. Also, identification of any "control" protein samples used for comparison to test samples.
3. Explanation/identification of the additional bands present in the Mouse BBMV samples.
4. Further discussion and justification regarding the "displacement" of the labeled Cry9C proteins by the unlabeled proteins.

**Bt Cry9C Protein Mouse Acute Intravenous Toxicity Study - MRID 447343-02**

An acute intravenous toxicity study was conducted with proteins derived from two *B. thuringiensis* species and from BSA. From the data presented, there do not appear to be toxic effects associated with any of the three proteins. A 14-day observation period after dosing did not reveal any abnormalities associated with injection of any of the proteins.
CLASSIFICATION: ACCEPTABLE

**Mouse Short-Term (30 Day) Dietary Toxicity Study with the Protein Cry9C - MRID 447343-03**

The test substance was provided to the test animals in their water at doses from 0.21 to 2.1 gm Cry9C/liter with a calculated daily water consumption of 5.0 ml/30.0 gm mouse. All animals appeared healthy, survived to termination of the study, and generally gained weight. No significant differences were seen in the parameters measured for clinical chemistry or hematology. The pathologist reported increased leanness in high dose treated mice seen in both the superficial tissues and abdominal viscera (10/12 animals). The hearts in the treated groups were also noted as having surface hemorrhages in 5/12 and 9/12 animals in the low and high dose Cry9C treatments, respectively. No unusual histopathology findings were made of any of the tissue

sections examined. The immunocytological examination of the GI tract found no binding of the Cry9C protein to villi or enterocytes lining the crypts of both the large and small intestines. Lymphatic tissue of the intestines (i.e., Peyer's patches), the spleen, submandular glands, mesenteric lymph nodes and thymus were all normal upon microscopic examination. Western blot analysis of fecal contents did find Cry9C in a degraded form (55kDa). There was an apparent change in the fat content of the high Cry9C dose group which was seen in a subjective determination of decreased fat pad size. The 28 day (and only) urinalysis of the high dose Cry9C group also indicated an elevated ketone level. Both these findings indicate some perturbation in the fat metabolism of the high dose group which has unclear toxicological significance.

CLASSIFICATION: Acceptable. The dietary exposure study was not required for this active ingredient.

**Determination of the Stability of PAT and Cry9C Protein in Processed Grain of Transgenic Corn in Fractionated Agricultural Commodities - MRID 447343-04**

Cry9C protein expression data was generated from whole and processed corn samples collected from transgenic and non-transgenic corn plants. The data presented in Tables 2, 3 & 4 indicate the amount of the PAT and Cry9C proteins present in the respective parts of the corn and corn

products. Table 4 provides relative information regarding the amounts of each of these proteins, and their amounts as a percentage of total proteins in the representative materials. Overall, based upon the data provided, these proteins are present at a maximum percentage of 0.0685% (dry mill - solvent extract germ), representing a relative small amount of total protein.

However, these data are somewhat questionable due to the levels of proteins found in the control samples grown in Illinois. It is certainly odd that both proteins are found in many of the control samples. It is possible that these results are simply the result of contamination of the control corn samples either in the field, or during the processing phase of the study. The validation assay was carried out using samples from a control plot of Glufosinate resistant corn grown in North Carolina (1997). The data and analysis from this study appear to be adequate; however, because the control was a different line of corn, grown in a different state under different (unidentified) growth conditions, these data can also be considered questionable in their relevance to this study. In addition, because they do not address the issue of why the control samples gave positive results for the proteins in question, the data do not appear to resolve the issue of the Illinois-grown controls.

As they are presented, the overall numbers do support the suggestion by AgrEvo that the Cry9C and PAT proteins represent a relatively small amount of the total proteins found in the transgenic plants. However, this is based upon the assumption that the titers of the proteins provided in this report are accurate. Because the control samples did show positive signals for each proteins, the accuracy of these numbers is questionable.

CLASSIFICATION: SUPPLEMENTAL. This submission can be upgraded to ACCEPTABLE with submission an adequate explanation for why the control samples also showed positive ELISA results for the PAT and Cry9C proteins, or supplemental data to address this issue.

**Development of New Methods for Safety Evaluation of Transgenic Food Crops - MRID 447140-02**

The results of intraperitoneal injection of corn powder extracts into BN rats indicate that both the control and transgenic corn powders are able to induce IgE or reagininc antibody responses by the PCA assay. The use of corn powder immunogen decreases the rate of the immune response to the Cry9C protein compared to the bacterial preparation. However, the lowest responding dose for Cry9C was similar for the two preparations (between 0.1 and 0.4 µg Cry9C). The control challenge test with the heterologous antigen of control corn powder or transgenic corn powder in the day 42 sera samples indicated that there was significant reactivity from the corn portion of the extracts themselves in the PCA assay. It is unclear, given this background reactivity, how conclusions can be made about the reactivity of the Cry9C protein alone. The PCA results from oral sensitization with ovalbumin II, control corn extract, bacterial Cry9C and transgenic corn (apparently supplemented with bacterial Cry9C) indicated that an IgE or reagin antibody response was elicited in naïve Sprague-Dawley rats. Ovalbumin sensitized serum produced a low frequency of responders and a weak dose response between the 5.0 and 50.0 mg/kg dose levels on days 28 through 42. The control corn also produced a positive oral sensitization response but this was only examined at the 50 mg/kg dose. Oral dosing with bacterial Cry9C gave a positive PCA response as did the Cry9C amended transgenic corn extract. The frequency of response to bacterial Cry9C began to diminish in day 42 sera. The Cry9C amended transgenic corn had a higher frequency of responders and the frequency remained high on day 42 PCA response. Western blot analysis indicated that Cry9C protein bands could be recognized in the rat sera from both exposure routes.

CLASSIFICATION: Supplemental. A more detailed description of the materials and methods, especially how the PCA system was utilized in these experiments, is needed. Specifically: (1) how long after the serum injections were the challenge antigen injections done; (2) what was the concentration of Cry9C in the bacterial and corn plant powder preparations; (3) The source of the test animals and their care during the test were not described; (4) How many naïve Sprague Dawley rats were used for each serum tested; (5) What were the individual animal responses; and (6) The original gel for the western blot assay should be provided.

**Occupational Exposure of Starlink Corn: Garst Seeds, 1996-1998 - MRID 447140-03**

The testimonial letters submitted by employees of the Garst Seed Company indicate that 1980 people with considerable direct exposure to corn seed and plant parts including tassels and pollen have not experienced adverse or allergic responses they could directly attribute to exposure to the Cry9C protein in StarLink corn.

CLASSIFICATION: Acceptable. The company is reminded that they are still responsible for reporting any incidents of hypersensitivity or other adverse effects they are aware has resulted from exposure to the Cry9C protein.

**Assessment of the Stability to Digestion and Bioavailability of the LYS Mutant Cry9C**

**Protein form *Bacillus thuringiensis* serovar *tolworthi* - MRID 447343-05**

Two separate studies are reported here. One is an *in vitro* stability assessment of the Cry9C protein; the other an *in vivo* assessment of bioavailability of Cry9C protein after gastric intubation of cannulated rats. All three batches of Lys mutant Cry9C were shown by SDS-PAGE and western blot to be substantially made up of Cry9C protein compared to the 100% standard provided by PGS. Greater than 90% of each preparation had the 68 kDa band as the major component with the majority of the remaining contaminant being the 55 kDa degradation form. The three batches of Lys mutant Cry9C either from *E.coli* (batches I & II) or *B. thuringiensis* (batch III) were shown to be substantially resistant to degradation after 2 hours by either SGF or SIF. When the Cry9C samples were subject to heat (either room temperature 20˚C or 90˚C) for extended periods the protein appeared to be stable as the protein banding pattern in SDS-PAGE gels is unaltered. The presence of a tomato matrix does not appear to affect this heat stability. However, the company has presented an isoelectric focusing gel which purports to show a loss of detectable protein forms after 30 to 60 minutes.

The data for bioavailability of Cry9C protein was monitored by the indirect double antibody sandwich ELISA previously described which had a detection limit of 2.0 ng Cry9C/ml in serum with a recovery rate of 85±5%. Previous work with Cry1Ab5 is also presented and that ELISA had a detection limit of 5.0 ngCry1Ab5/ml with a recovery rate of 55±5%. The animals were dosed with a range of Cry9C from 2.6 to 298 mg/kg bodyweight by gastric intubation. All the dosed animals appeared and behaved normally, survived to sacrifice and displayed no treatment related adverse effects. No ELISA positive Cry9C protein or fragments were detected in blood samples taken from rats at the 2.6mg/kg dose. At the higher dose rates, Cry9C ELISA values were positive giving a calculated value of between 5 and 15 ng Cry9C/ml in serum. The absorption rate was not affected by dose amount or presence of a food matrix. The percentage of bioavailable Cry9C was calculated to range from 0.0006% (42mg/kg) to 0.002% (298mg/kg) of the dose administered. More importantly, an SDS-PAGE/western blot confirmation of the sera that were ELISA positive for the presence of Cry9C protein showed that only one of the rats had serum with confirmed Cry9C protein. This rat (#548) had a faint Cry9C positive band, all the other sera had no detectable Cry9C reactivities. No interfering compounds were found in rat serum or BSA. The positive signals were attributed to cross-reaction with Cry9C like components present in both control and Bt-treated animals since there were no significant differences between the 2-D electrophoresis or ECL results. This data indicating cross reaction was not presented. CLASSIFICATION: Acceptable.

**The effect of corn hybrid CBH351 on the growth of male broiler chickens. - MRID No. 447343-06**

This is not a required study and the results are inconclusive. Small differences were seen with the CBH351 test groups as compared to the the non-CBH351 test groups. Increased feed intake during the starter period, an increase in bird weight, and greater breast meat yield were observed. However, it is not possible to make an independent assessment of the significance of the data without an analysis of the bird feed for the presence of Cry9C protein. **ADEQUACY OF STUDY:** 1. Validation category: Not useful for avian diet effects assessment. 2. Rationale: The

submitter does not claim GLP compliance, no Quality Assurance statement is included with the study report, and the report lacks analysis for the presence of Cry9C in the test diet.

**The following data were submitted in support of the currently approved feed use only exemption from the requirement of a tolerance.**

***In Vitro* Digestibility and Heat Stability of the Endotoxin Cry9C of *Bacillus thuringiensis* - MRID No. 442581-08**

The samples of lyophilized Cry9C protein expressed in corn showed no signs of protein disintegration when subjected to *in vitro* digestion in simulated mammalian gastric fluid. These digestions were done either with of without pepsin in the low pH buffer and were assayed by western blot from samples taken at several time points from the mixing the reagents to after 4 hours exposure to the digestive fluids. The same 70kD double band seen in the original Cry9C protein in plant tissue at time 0 was also seen, undiminished , in all the subsequent incubation samples. No effect on Cry9C activity as determined by bioassay was seen after any heat treatment. The most stringent heat treatment was 90°C for 10 minutes. CLASSIFICATION: ACCEPTABLE.

**Amino Acid Sequence Homology Search with the Corn Expressed Truncated Cry9C Protein Sequence - MRID No. 442581-09**

Three hundred sequences were listed as having regions of homology with the 626 amino acids of the Cry9C truncated toxin protein. The first 64 proteins in the list were all parasporal proteins from *Bacillus thuringiensis* otherwise known as δ-endotoxins. Other δ-endotoxins were found at 67, 76, 78, 79, and 80. These proteins had regions of homology that gave a "significant homology". The table of values indicated a matching score above 4 standard deviations would contain all the δ-endotoxins mentioned above. The algorithim for converting the matches and penalties into homology scores was not described although it was stated that " all other proteins (besides the δ-endotoxins referred to above) have less than 20% exact sequence matching and no major stretches of sequence homology could be detected, indicating that in these cases the sequence homology is not significant." CLASSIFICATION: SUPPLEMENTAL. BPPD has not yet finalized requirements regarding amino acid sequence homology for risk assessment purposes. No further studies need to be done at this time for analyzing homology.

**Cry9C Bacillus thuringiensis Insecticidal Protein Identification of Sequence Homology with Allergenicity by Searching Protein Databanks - MRID No. 443844-04**

Sequence identity for any of the eight amino acid regions in Cry9C was found only to other Bt crystal proteins. No match between any 8 amino acid sequence in Cry9C and any of the allergenic

proteins known in the SWISS protein database was found. This lack of homology at a finer level of examination is further evidence that Cry9C is not related to known allergens using a structural consensus approach. CLASSIFICATION: SUPPLEMENTAL. BPPD has not yet finalized requirements regarding amino acid homology for risk assessment purposes. No further studies need to be done at this time for analyzing homology.

**An Acute Oral Toxicity in Mice with Cry9C Protein as Purified from *Bacillus thuringiensis* Cry9C.PGS2 - MRID No. 442581-07**

There were no deaths in any test animals due to test material given at the dose of 3,760 mg/kg during the 14 day observation period. One male mouse displayed hair loss between days 2 and 5. One female displayed decreased activity on the day of dosing. Another female displayed decreased activity , wobbly gait, decreased feces and felt cool to the touch. A third female displayed decreased feces on day 1. All the male mice gained weight during the test period (except during the pre-dosing fast period). Two female mice failed to gain weight between day 0 (prefasted weight) and day 7 and three failed to gain weight between day 7 and day 14. One female did not recover her pre-fasting body weight by day 14. CLASSIFICATION: Acceptable.

**Investigation of Allergens in Wild-Type and Transgenic Corn - MRID No. 443844-05**

The 21 sera samples from suspected corn-sensitive individuals all tested positive in the RAST assay by having >3% reactivity. The transgenic and wild-type aqueous corn extracts were not obviously different in responsiveness for individuals and a t-test of the RAST % reactivity did not reveal any significant differences. The RAST inhibition assay gave results indicating that both wild type and transgenic corn extracts gave substantial inhibition of the wild type corn RAST. Statistical analysis of the inhibition curves generated for RAST inhibition from wild type versus transgenic corn extracts did not indicate significantly different 50% inhibition values, slopes or y-intercepts. The type of extract, aqueous or alcoholic, utilized in the inhibition assays was never specified. Both the wild type and transgenic aqueous corn extracts gave higher levels of reactivity in the immunoblot assay than the alcoholic extracts. A comparison of the IgE reactions for specific corn atopic individuals indicated that there were similar reactive banding patterns in both transgenic and wild type corn. In some individuals there were a greater number of reactive bands ranging in molecular weight whereas in others there were only one or two bands, generally of lower molecular weight, which had very significant staining. There was no identification of individuals in the SDS-PAGE lanes so no correlation between the intensity of the % reactivity in RAST and the number or intensity of staining in the immunoblot assay could be made. A two-fold dilution series with a pool of 10 RAST positive corn atopic sera was tested against the wild type and transgenic corn extracts. The pattern of reactivity was very similar between the transgenic and wild type extracts with the intensity of the reaction again being higher for the aqueous versus alcoholic extracts. There were some unique bands present in either the wild type or transgenic extracts but since these bands did not show detectable effects on the serum reactivity kinetics in the RAST or RAST inhibition assays it is difficult to judge the importance of their presence. CLASSIFICATION:SUPPLEMENTAL. This study does not address the potential for inducing food allergy from a novel protein lacking a history of dietary exposure. An additional control testing purified Cry9C protein against corn atopic sera should have been

included to establish the negative reactivity background. The study does establish a baseline of corn allergen reactivity for subsequent comparisons if such an allergic response does occur over time.

**Preparation and characterization of catfish pellets - MRID No. 443843-01**

Based on results of a protein-specific ELISA analysis, no Cry9C protein was detectable in catfish pellets processed from corn kernels containing Cry9C protein.

**EPA Overall Conclusion Regarding Data Submitted by AgrEvo**

AgrEvo has provided significant amounts of data to address the question of potential food allergy. Food allergy itself is a research field currently in transition from descriptive clinical symptomatology to the more analytical aspects of epitope mapping known food allergens and developing valid animal models of food allergy. Unfortunately, the process of food sensitization, hence potential food allergenicity, is an area of food allergy that is still very little understood. The supplementary data supplied by AgrEvo do not aid substantially in confirming or refuting the supposition that Cry9C is a potential food allergen. The indications that Cry9C is not substantially altered by incubation in gastric or intestinal fluids were confirmed. The gastric incubation did degrade about 15 to 25% of the protein as measured by scanning densitometry compared to the controls. In dosed animals the 68kDa form of Cry9C is converted to a 55kDa form, perhaps by the microbial action of the intestines, but no further degradation is evident. No significant uptake into the blood of any immunoreactive form of Cry9C is indicated by the bioavailability study. The 30-day dietary study presented no adverse effects, except a change in the fat pads of animals in the high dose groups and an altered level of ketone in the blood which may be indicative of altered carbohydrate metabolism. The brown Norway rat study indicated that the Cry9C, Cry1Ab, lactoferrin (not to be considered of clinical importance in milk allegies), and other proteins were recognized as food allergens by sensitized test animals orally challenged with that same protein. The brown Norway rat model is not a validated test for food allergy at this point and the study submitted is significantly flawed by contamination of the corn tissue controls, poor description of the experimental protocols and possibly immunization procedures themselves. The protein expression data are questionable due to apparent contamination of the controls.

## IV Questions That EPA is Presenting for Comment in Addition to the Cry9C Reviews

Cry9C - Specific Questions:

1. Is the brown Norway rat study an animal model of food allergy recognized as valid and useful by the scientific community? Has the model been validated by recognizing known food allergens and not recognizing other dietary proteins? Is the use of an adjuvant such as carrageenan appropriate to examine a normally functioning immune system?

2. Does the bioavailability study provide useful information about allergic potential? Can a protein be a food allergen without being able to cross the GI mucosa? Is gut permeability

too variable within the population to be used as a screening tool?

3. In the case of the Cry9C protein, does the apparent degradation of the 68 kDa protein to a 55 kDa protein suggest anything regarding the digestibility/allergenicity of this protein?

4. Does the additional data provided by AgrEvo either reduce or alleviate concern of Cry9C as a potential allergen or further implicate Cry9C as a potential allergen?

Overall Protein Allergenicity:

Considering the conclusions drawn from the "Conference on Scientific Issues Related to Potential Allergenicity in Transgenic Food Crops," April 18-19, 1994 in Annapolis, Maryland discussed above in Section II:

5. Are the characteristics of heat stability and resistance to digestive enzymes useful criteria to screen for food allergenicity? Are there known examples of dietary proteins that have these stability characteristics yet are not allergenic?

6. Does the lack of amino acid homology offer predictive function to examine the allergenicity of a new dietary protein, or alternatively does it simply indicate which allergic population should be examined to look for possible reactivity?

7. There is anecdotal evidence that total dietary exposure to a food correlates to food allergy (i.e., prevalence of rice allergy in eastern Asia, fish allergy in Scandinavia, wheat allergy in Europe and the Americas). Does level of exposure in the diet affect the sensitization phase of food allergy? Would exposure to a protein as a minor component of direct dietary consumption lessen the likelihood a protein would either be or become a food allergen? Is there evidence that feed exposure (i.e., soybean meal as an animal feed) can affect the allergenicity of the resulting meat, milk or eggs? For example, does the use of soybean meal as animal feed make the resulting meat, milk or eggs an allergenic risk for a soybean sensitive individual?

8. Is it feasible to monitor changes in the incidence of human food allergy to stable proteins? How quickly are newly introduced food allergens typically identified after they first become part of the human diet?